

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Yanmin Zhao, Jimin Shi, and He Huang designed the study and supervised the analyses and manuscript preparation. Yanmin Zhao, Fei Gao, and Yang Gao collected and analyzed the data, Fei Gao and Yang Gao wrote the manuscript. All other authors discussed and interpreted the results. All authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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Identification of recurrent genomic alterations in the apoptotic machinery in chronic lymphocytic leukemia patients treated with venetoclax monotherapy

To the Editor:

The anti-apoptotic protein B-cell lymphoma 2 (BCL-2) is a key regulator of the intrinsic apoptotic pathway,¹ and survival dependence on BCL-2 has been demonstrated in chronic lymphocytic leukemia (CLL).² Venetoclax is a highly selective, potent, orally bioavailable

[Correction added on January 29, 2022, after first online publication: Copyright was changed.]

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BCL-2 inhibitor³ that has demonstrated efficacy as monotherapy for patients with previously treated CLL in four clinical studies.^{4–8} Because venetoclax is the first approved BH3 mimetic and an approved treatment option for CLL, strong interest exists in learning about potential mechanisms of resistance.

To investigate resistance to venetoclax in vitro, we performed a genome-wide CRISPR knockout screen. In the absence of a suitable CLL cell line, it was performed in SU-DHL4, a diffuse large B-cell lymphoma cell line. Upon venetoclax treatment, cells with potential genetic perturbations leading to drug resistance were enriched in the surviving population. Consistent with previously described functional genomic screens across multiple disease models,⁹ genes encoding members of the pro-apoptotic machinery, including *BAX*, *BCL2L11* (*BIM*), and *PMAIP1* (*NOXA*), were among the strongest hits associated with venetoclax resistance (Figure 1A). Loss of *ID3* or *NFKBIA* was also associated with decreased venetoclax sensitivity, suggesting multiple possible mechanisms of resistance.¹⁰

To compare these results with potential mechanisms of resistance in patients treated with venetoclax, we performed whole-exome and targeted sequencing of DNA extracted from CD19+ B cells collected before treatment initiation and/or upon treatment discontinuation from 43 patients with relapsed/refractory CLL or small lymphocytic lymphoma. All patients were enrolled on a venetoclax monotherapy study (NCT01328626,⁵ $n = 18$ or NCT01889186,⁶ $n = 25$) and received treatment until disease progression or unacceptable toxicity. Selection for these analyses was based on sample availability. Baseline characteristics are shown in Table S1.

Median time on therapy was 20.1 months (range, 1.2–60.3) with a median duration follow-up of 61.3 months (95% CI, 48.6–73.4). Venetoclax was nearly as efficacious in this cohort as it was in the overall cohort^{5,6} (and pooled data of 347 patients across three venetoclax studies¹¹), with an overall response rate (ORR) of 69.8%, a complete remission/complete remission with incomplete hematopoietic recovery (CR/CRi) rate of 11.6%, and median progression-free survival (PFS) of 21.9 months (Table S2). Although minimal residual disease (MRD) evaluation was not required for all patients, of the 12 patients with ≥ 1 MRD assessment, five achieved undetectable MRD at 10^{-4} in the peripheral blood, whereas seven had detectable MRD. Post-treatment specimens were collected at study discontinuation due to CLL progression ($n = 29$), Richter's transformation ($n = 8$), or other reasons ($n = 6$) (Table S3).

To investigate changes in clonal architecture after venetoclax treatment in CLL, we focused on three of the most mutated genes in CLL: *NOTCH1*, *SF3B1*, and *TP53* (Figure 1B). Although some gains and losses of mutations in these genes were observed, most mutations present before treatment initiation were detected in post-venetoclax specimens. Gains and losses of *NOTCH1* mutations were detected in post-treatment samples of four and two patients, respectively. One patient's CLL gained an *SF3B1* mutation at progression, whereas in three patients, *SF3B1*-mutated clones present at commencement were no longer detected. We did not observe gains of *TP53* mutations. For three patients, *TP53*-mutant clones present at commencement were not detected subsequently, supporting the notion that venetoclax-based treatments are efficacious in CLL

patients with these mutations. Two patients gained *BRAF* V600E mutations, consistent with a recent report.¹² Overall, based on the markers evaluated, one-third ($n = 14/43$) of patients exhibited changes in clonal architecture during treatment.

Previous studies have identified *BCL2* mutations associated with relapse on venetoclax.^{13–15} In our cohort, no *BCL2* mutations were found at study entry, but *BCL2* mutations were acquired in CLL cells from 15 of 43 patients (35%), 14 of whom experienced CLL progression (Figure 1C). Of 29 patients with CLL progression, *BCL2* mutations were identified in 10 patients who were on treatment for ≥ 24 months. Overall, 30 distinct *BCL2* mutations were identified, with multiple mutations identified in nine patients (Figure 1D,E). The most frequent mutations of any description observed were at residue D103 (11 occasions) and G101 (8 occasions). Additionally, mutations were identified at F104 (one occasion), A113 (three occasions), and V156 (four occasions). A 4--amino acid (RRYR) insertion at residue 110 was identified in samples from three patients. Except for the insertion, all *BCL2* alterations were missense mutations. In agreement with previous findings,^{13,14} most *BCL2* mutations were found in minor clones (Figure 1F). Only 1 *BCL2* mutation (D103Y) had a variant allele frequency (VAF) $> 20\%$, whereas most *SF3B1* and *TP53* mutations were in larger clones (VAF $> 20\%$).

Mutations in apoptosis regulators other than *BCL2* were identified, including *PMAIP1* and *BAX*, two of the strongest hits identified in the CRISPR screen. All *PMAIP1* mutations were loss-of-function, including three nonsense mutations and one mutation leading to loss of translation-initiating methionine, M1. One *BAX* mutation was nonsense; the other mutation replaced leucine at amino acid 181 with histidine. Although both *BAX* mutations had a VAF $< 10\%$, three of four *PMAIP1* mutations had a VAF $> 20\%$, suggesting that *PMAIP1* mutations could perhaps be a key driver of venetoclax resistance, although further study is needed. In addition to a *BCL2* mutation, one patient also had a mutation in the pro-apoptotic gene *BAD* (G111R) with a VAF $> 25\%$; the functional relevance of this is unknown. Three *PMAIP1* mutant patients also had *BCL2* mutations present at the end of treatment, albeit at different allele frequencies, supporting the idea of multiple potential mechanisms of resistance within an individual patient.

Although initial clinical response to venetoclax-based therapies are similar in CLL patients regardless of *TP53* disruption via *del(17p)* and/or *TP53* mutation, patients with *del(17p)/TP53* mutation had shorter response duration.^{11,16,17} In our cohort, 78% of patients ($n = 29/37$) with CLL progression or Richter's transformation had a *del(17p)/TP53* mutation at baseline. Additionally, 87% of patients ($n = 13/15$) with *BCL2* mutations had evidence of *TP53* disruption.

To provide a deeper understanding of the characteristics of the patients available for these exploratory analyses, the outcomes are reported. Among the eight patients who progressed with Richter's transformation, the ORR was 25.0% with a median PFS of 5.3 months (Table S2). While two patients achieved partial remission (PR), most patients were refractory to venetoclax prior to diagnosis of Richter's transformation. One patient who acquired a *BCL2* mutation achieved a PR but relapsed after 6 months. In contrast, the ORR for patients who eventually relapsed due to CLL

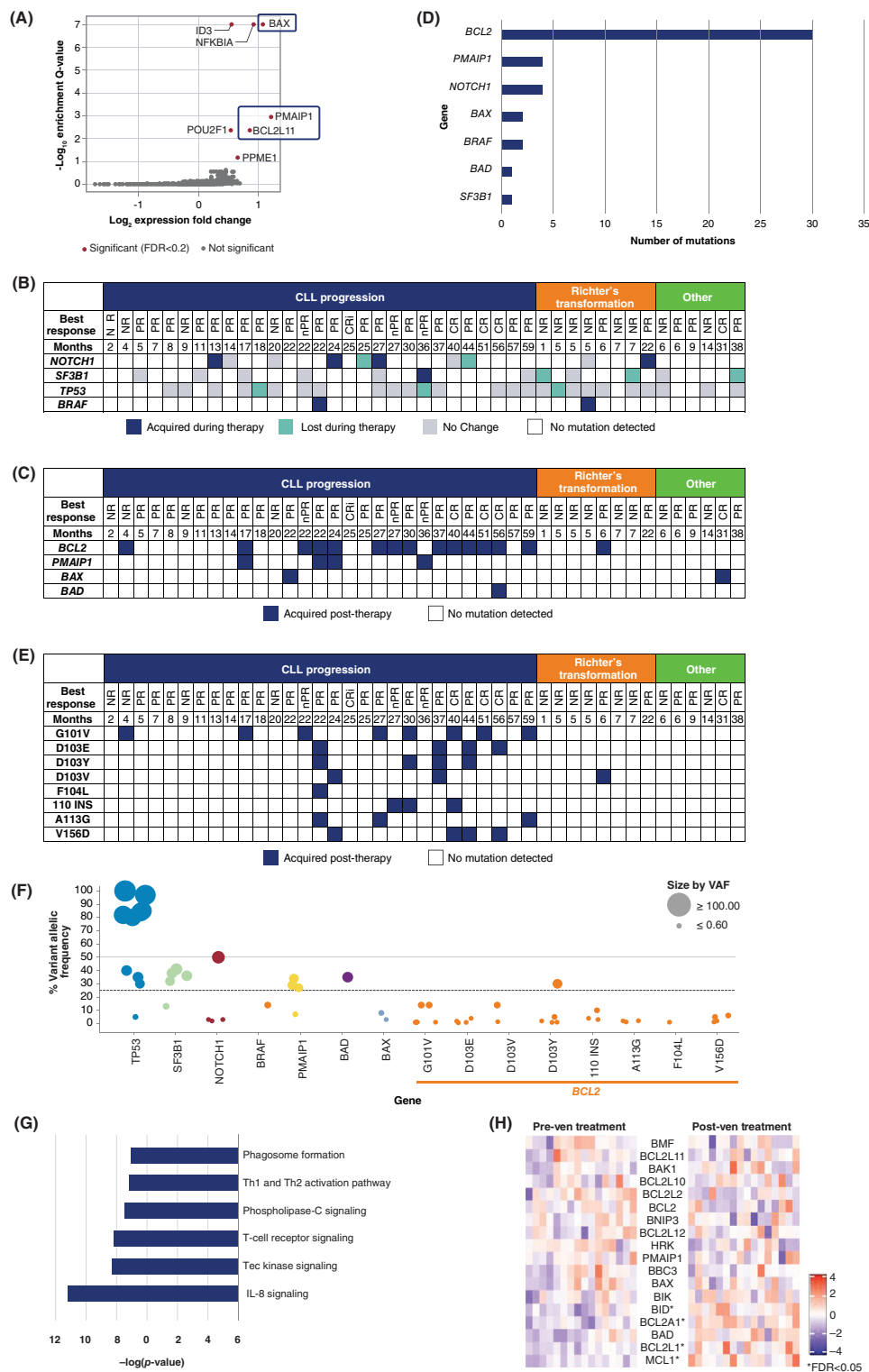


FIGURE 1 (A) Gene deletions identified from a pooled genome-wide CRISPR screen in SU-DHL4 cells that confer venetoclax resistance. (B) Tumor genomic changes in patients with CLL after venetoclax monotherapy. (C) Acquired mutations in apoptotic machinery after venetoclax monotherapy. (D) Frequency of acquired mutations after venetoclax monotherapy. (E) Acquired *BCL2* mutations after venetoclax monotherapy. (F) Variant allele frequency of mutations. (G) Upregulation of genes in patients who relapsed on venetoclax monotherapy. (H) Changes in expression of *BCL2* family genes pre- and post-treatment. * represents significant expression changes (FDR < 0.05). CR, complete remission; CRi, complete remission with incomplete hematopoietic recovery; nPR, nodular partial remission; NR, nonresponder; PR, partial remission; VAF, variant allele frequency

progression was 86.2%, with seven achieving a deep remission (CR/CRi/nodular partial remission) and 18 achieving a PR. Median duration of response (DOR) was 23.7 months and median PFS was 21.9 months. Among patients who relapsed with CLL progression, median PFS for patients with a *BCL2* mutation at relapse was 27.3 months but for patients without a *BCL2* mutation median PFS was 10.8 months. Similar results were demonstrated for patients with a mutation in any apoptotic gene; median PFS for patients with versus without an acquired mutation in any apoptotic gene was 27.3 and 10.8 months, respectively. Lastly, for patients who acquired a mutation in *BCL2* or other apoptotic genes, the DOR was longer compared with DOR in patients without a mutation (Table S2). Given the observation that acquisition of mutations in apoptotic genes did not portend a shorter response, we evaluated gene expression changes in the pre- and post-progression specimens.

To investigate transcriptional changes that occurred, we performed RNA-seq on 16 pairs of pre- and post-venetoclax samples from patients with CLL progression. Comparison of samples identified changes in expression of over 800 genes (FDR < 0.05). Several genes that play a role in IL-8, Tec kinase, and phospholipase C signaling were upregulated in patients at relapse (Figure 1G). Comparison of pre- and post-treatment samples between patients who were on study for different durations of time did not yield any differentially expressed genes. We were interested in looking at changes in expression of *BCL2* family genes because they are known to be able to affect venetoclax resistance in vitro (Figure 1H). A trend of decreased *BCL2* expression in post-venetoclax samples was observed, although the differences did not reach statistical significance. Concomitantly, we observed a statistically significant increase in expression of *MCL1*, *BCL2L1* (*BCL-X_L*), and *BCL2A1* (*BFL-1*), three genes whose protein products can inhibit apoptosis when *BCL-2* is effectively targeted by venetoclax.

In addition to previously published mutations in *BCL2*, we identified novel mutations in *PMAIP1*, *BAX*, and *BAD* in CLL patients who developed resistance to venetoclax monotherapy. This is the first confirmation of the top hits from an in vitro screen with patient samples from clinical trials. Our findings suggest that inactivation of *BAX*, *BAD*, or *PMAIP1* may be sufficient to render CLL cells resistant to venetoclax treatment, identifying these factors as potential critical regulators of venetoclax response. This is the first report to evaluate mRNA expression differences in this patient population. Observed gene expression changes indicated that tumor cells may upregulate anti-apoptotic *BCL-2* proteins other than *BCL-2* to avoid apoptosis induction by venetoclax. Overall, our data suggest that resistance to venetoclax monotherapy in CLL patients is multifactorial and highly dependent on mutational and gene expression changes that alter the balance between the pro- and anti-apoptotic machinery. Additional studies are needed to determine the clinical relevance of minor *BCL2* mutant clones in venetoclax resistance and whether similar mechanisms of resistance arise in patients treated with fixed-duration venetoclax combination therapies.

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CONFLICT OF INTEREST

R.P., F.D., C.L., K.R., D.Q., S.E.W., N.M., A.J.S., J.F.W., J.A.R., and B.J.C. are employees of AbbVie and may hold stock or other options; J.P. is a former employee of AbbVie, current employee of GlaxoSmithKline, and may hold stock or other options with AbbVie; M.S.D. received institutional research grants from AbbVie, Ascentage Pharma, AstraZeneca, Genentech, MEI Pharma, Novartis, Surface Oncology, TG Therapeutics, and Verastem; and personal fees from AbbVie, Adaptive Biotechnologies, Ascentage Pharma, AstraZeneca, BeiGene, Bristol Myers Squibb, Celgene, Eli Lilly, Genentech, Janssen, MEI Pharma, Merck, Novartis, Research to Practice, Takeda, TG Therapeutics, and Verastem; E.T. received fees for consulting and research support from AbbVie, Janssen, and Roche; S.S. received fees for consulting, drug/equipment, funding, and writing assistance from AbbVie, AstraZeneca, Celgene, Gilead, GlaxoSmithKline, Hoffman La-Roche, Janssen, Novartis, Pharmacyclics, and Sunesis; J.D.L. is a former employee of AbbVie, current employee of Pfizer, and may hold stock or other options with AbbVie; S.Y.K. is a former employee of AbbVie, current employee of Nurix Therapeutics, and may hold stock or other options with AbbVie.




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DATA AVAILABILITY STATEMENT

AbbVie is committed to responsible data sharing regarding the clinical trials we sponsor. This includes access to anonymized, individual and trial-level data (analysis data sets), as well as other information (e.g., protocols and Clinical Study Reports), as long as the trials are not part of an ongoing or planned regulatory submission. This includes requests for clinical trial data for unlicensed products and indications. This clinical trial data can be requested by any qualified researchers who engage in rigorous, independent scientific research, and will be provided following review and approval of a research proposal and Statistical Analysis Plan (SAP) and execution of a Data Sharing Agreement (DSA). Data requests can be

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Sutimlimab, an investigational C1s inhibitor, effectively prevents exacerbation of hemolytic anemia in a patient with cold agglutinin disease undergoing major surgery

To the Editor:

Cold agglutinins (CAs) are autoantibodies, usually of the IgM class, directed against surface proteins expressed on red blood cells (RBCs). Most CAs are of low clinical significance since their thermal